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# Identification of Small Molecules against Botulinum Neurotoxin B Binding to Neuronal Cells at Ganglioside GT1b Binding Site with Low to Moderate Affinity

Nagarajan Pattabiraman
Carolyn Chambers
Ayesha Adil
Gregory E. Garcia

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**US Army Medical Research Institute of Chemical Defense**
**3100 Ricketts Point Road**
**Aberdeen Proving Ground, MD  21010-5400**

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**14. ABSTRACT**

*Clostridium botulinum* bacteria produce botulinum neurotoxin (BoNT) which can be developed as a bioweapon. Currently no FDA-licensed prophylactic product is available in the USA against botulism. Small molecules are being developed for use as inhibitors against the proteolytic activity of the toxin, but we are investigating an alternative strategy where small molecule inhibitors may prevent intoxication by disrupting the binding process of the toxin to neuronal cells. Recently, four potential binding sites or pockets on BoNT serotype B (BoNT/B) for the trisaccharide GT1b were identified from the x-ray crystal structure of the BoNT/B/trisaccharide (GT1b) complex (PDB id: 1F31). To develop inhibitors for these sites we designed a five-point 3D stereo-electronic pharmacophore model for Pockets 1 and 2. Queries were made of compound libraries from the NCI/DTP (National Cancer Institute/Developmental Therapeutics Program) based on features of the pharmacophore model. Library compounds were screened *in silico* to identify those that could dock into Pocket 1 or a combination of Pockets 1 and 2. We rank-ordered the compounds based on consensus scoring. Seventy compounds were identified by the model. Available compounds were tested in a cell-based ELISA binding inhibition assay. With this assay we identified two lead compounds that prevented the binding of BoNT/B binding domain (BD) to GT1b. These initial compounds demonstrate the utility of this approach and could form the basis of further discovery and refinement for the design of more potent small molecule inhibitors.

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**ABSTRACT**

*Clostridium botulinum* bacteria produce botulinum neurotoxin (BoNT) which can be developed as a bioweapon. Currently no FDA-licensed prophylactic product is available in the USA against botulism.  Small molecules are being developed for use as inhibitors against the proteolytic activity of the toxin, but we are investigating an alternative strategy where small molecule inhibitors may prevent intoxication by disrupting the binding process of the toxin to neuronal cells.  Recently, four potential binding sites or pockets on BoNT serotype B (BoNT/B) for the trisaccharide GT1b were identified from the x-ray crystal structure of the BoNT/B/trisaccharide (GT1b) complex (PDB id:  1F31). To develop inhibitors for these sites we designed a five-point 3D stereo-electronic pharmacophore model for Pockets 1 and 2.  Queries were made of compound libraries from the NCI/DTP (National Cancer Institute/Developmental Therapeutics Program) based on features of the pharmacophore model. Library compounds were screened *in silico* to identify those that could dock into Pocket 1 or a combination of Pockets 1 and 2. We rank-ordered the compounds based on consensus scoring. Seventy compounds were identified by the model. Available compounds were tested in a cell-based ELISA binding inhibition assay. With this assay we identified two lead compounds that prevented the binding of BoNT/B binding domain (BD) to GT1b. These initial compounds demonstrate the utility of this approach and could form the basis of further discovery and refinement for the design of more potent small molecule inhibitors.

**INTRODUCTION**

Botulinum neurotoxins (BoNTs) are the most poisonous substances known (Schiavo et al., 1994) and can be developed as bioweapons. Thus far research into medical countermeasures for BoNTs has focused on the development of vaccines, and the current standard of care is the use of a trivalent (A, B, E) botulinum antitoxin that was derived from equine (horse) sources utilizing whole antibodies. This antitoxin was only available from the local health department via the Centers for Disease Control and Prevention (CDC); however, its supply and shelf-life were limited. A pentavalent BoNT toxoid vaccine, which was manufactured in the 1970s and available under CDC IND-161, was discontinued by the CDC because of diminished potency and an increase in local reactions related to annual booster doses (Webb and Smith, 2013). Recently (March 2013), the FDA approved the first botulism antitoxin for use in neutralizing all seven known nerve toxin serotypes (A, B, C, D, E, F, and G). This heptavalent antitoxin product (BAT, Cangene bioPharma, Inc., Winnipeg, Manitoba, Canada) was manufactured from equine plasma. Side effects from this product include allergic reactions and delayed hypersensitivity reactions in persons who are sensitive to horse proteins. A recombinant bivalent vaccine (rBV A/B) expressed in *Pichia pastoris* entered Phase III clinical trials in 2013. Currently no FDA-licensed prophylactic product against botulism is available in the USA. In this project, we initiated a novel approach to develop a BoNT therapeutic specifically targeting entry of the BoNT into host cells.

*Clostridium botulinum* bacteria produce seven serotypes of BoNT. Each is released as a single polypeptide chain of about 150kDa, which when cleaved results in a 100kDa heavy chain (HC) and a 50kDa light chain (LC) linked through a disulfide bond (Singh, 2000). The mode of action of BoNT involves a three-step process: binding to the target cell surface, translocation of the toxin across the plasma membrane, and intracellular substrate cleavage resulting in the blockage of acetylcholine release. The HC contains the binding domain (BD) involved with cell surface interaction through binding to ganglioside and protein receptor. After binding, the N-terminal domain of the HC, the translocation domain (TD), is inserted into the membrane and acts as a passageway for the LC to enter the cytoplasm. The LC is a zinc–dependent endopeptidase that is responsible for the toxic activity of BoNT. After entering the cytoplasm via the TD, the LC cleaves one of three soluble N-ethylmaleimide–sensitive fusion (NSF) attachment protein receptor (SNARE) proteins (depending on the serotype): VAMP/synaptobrevin, SNAP-25, and syntaxin (Grumelli et al., 2005). This activity inhibits exocytosis, causing the failure of acetylcholine release at the neuromuscular junctions, which results in neuromuscular paralysis and death due to respiratory failure.

BoNTs initially bind to the negatively charged surface of the presynaptic membrane, which consists of disialogangliosides, trisialogangliosides and other acidic lipids with low affinity and then move laterally to bind to the specific protein receptor with high affinity (Montecucco, 1986; Kitamura et al., 1999). The ganglioside GT1b has been shown to bind to BoNT/B with low affinity (Nishiki et al., 1996; Kozaki et al., 1998). GT1b is a trisialo-ganglioside that is composed of ceramide linked by a glycosidic bond to an oligosaccharide chain containing hexose and N-acetylneuraminic acid, also known as sialic acid units. In 2000, the crystal structure of BoNT/B was solved (Swaminathan and Eswaramoorthy, 2000) and revealed that the BD has two sub-

domains, $H_{CN}$ (the N-terminal region of the heavy chain BD) and $H_{CC}$ (the C- terminal region of the heavy chain BD).

Rummel et al. reported that the protein receptor synaptotagmin (Syt) could potentially bind to four different pockets in the $H_{CC}$ (Rummel et al., 2007). Computer-based surface analysis using Molecular Operating Environment (MOE) Site Finder feature (Chemical Computing Group, Montreal, QC, Canada) and SuperStar version 1.5.1 (Cambridge Crystallographic Data Centre, Cambridge, U.K.) predicted hydrophilic and hydrophobic interaction sites and cavities. Site-directed mutagenesis studies confirmed that clusters of potential contact sites existed within hollows of the cavities. These hollows were called pockets of potential sites for binding of Syt. Figure 1 shows three of four pockets on the surface of the C-terminal region of the binding domain.

The identified pockets for interaction between the toxin and ganglioside GT1b or protein receptor binding site suggest a strategy for the prevention of intoxication by disrupting the binding process with the identification of small molecule inhibitors. Our investigation utilized *in-silico* screening of previously synthesized small molecule compounds from the National Institutes of Health National Cancer Institute Developmental Therapeutics Program (NIH NCI/DTP) in an attempt to determine if we could discover compounds that could be used to inhibit the binding of BoNT/B to neuronal cells. Further, one can identify analogs either from the commercially available chemical repository or by chemically modifying our lead compounds to develop QSAR for these compounds.

## MATERIALS AND METHODS

### Expression, purification and refolding of BoNT/B heavy chain recombinant protein:
The recombinant heavy chain domain of BoNT/B (HCD-BoNT/B) had been cloned into pET-15b vector by Zhou and Singh (2004). This clone contains a functional botulinum neurotoxin serotype B heavy chain translocation and binding domain (Ile 624-Glu1291) that produces an N-terminal six-histidine fusion protein. We received this clone as a gift from Dr. B. R. Singh. We expressed this clone in *E. coli* by induction using 1 mM IPTG (isopropylthio-β-galactoside). HCD-BoNT/B protein can be isolated by solubilization of *E. coli* inclusion bodies. The purified recombinant protein must be refolded after isolation by treatment with 6M guanidine-HCl in the presence of β-mercaptoethanol. The isolation, purification and refolding was conducted according to the conditions previously described by Zhou and Singh (2004) and modified by Schmidt et al. (2005). We routinely obtained 1 mg of recombinant HCD-BoNT/B protein purified and refolded from the expression in *E. coli*. This recombinant protein was used for subsequent biological ELISA binding assays. Total protein concentration of the refolded recombinant HCD-BoNT/B was determined using the Pierce 660 nm protein assay (Thermo Fisher Scientific, Waltham, MA).

### Compounds:
All small molecule compounds were obtained from the National Cancer Institute (NCI) Developmental Therapeutics Program (DTP) Open Chemical Repository as vialed samples (http://dtp.nci.nih.gov/branches/dscb/repo_open.html). DEP001 is a 21-residue peptide, 40-GESQEDMFAKLKEKFFNEINK-60, of the mouse Syt II (Dong et al., 2003) that was synthesized by Avanti (Alabaster, AL). GT1b was purchased from Sigma-Aldrich (St. Louis, MO). The mouse anti-His-Tag and goat anti-mouse IgG-AP conjugate antibodies were purchased

from EMD4Biosciences/Millipore (Billerica, MA). Aqueous soluble compounds were tested at least three times using the binding ELISA assay. Those compounds that were either insoluble in dimethyl sulfoxide (DMSO) or precipitated out of solution after the addition of buffer were not tested. Some compounds were not available.

***In-silico* screening:** The two pockets (-1, -1 & -2 combined) that have been identified in the heavy chain C-terminal sub-domain ($H_{CC}$) of the BD in the crystal structure of BoNT/B complexed with a trisaccharide were used for our *in-silico* screening of organic small molecule databases available from the NCI/DTP. Our approach was to use *in-silico* screening (Brooijmans and Kuntz, 2003) to screen compounds against Pocket-1 and also to screen compounds against the combined pocket formed by Pockets-1 and -2. *In-silico* screening was performed using MOE (Molecular Operating Environment, Chemical Computing Group, Canada). For each screening, consensus scoring based on energy interactions between the compound and the residues in the pocket and surface-based solvation was used to rank order the docking of these compounds. We selected 39 compounds for Pocket 1 and 25 compounds for Pocket 1-2 combined from *in-silico* screening for *in-vitro* testing.

**Immuno-dot blot assay for binding of synaptotagmin to purified BoNT/B recombinant HC domain:** We used an immuno-dot blot binding assay to monitor the binding of the HCD-BoNT/B to Syt II, as described by Thomas et al. (1999) and modified by Lalli et al. (1999) and Zhou and Singh (2004). Essentially, 1 μL of the HCBTBD batch sample (~0.2 mg/mL to 0.3 mg/mL) was spotted onto nitrocellulose, dried, and blocked (3% bovine serum albumin [BSA] in 50 mM sodium phosphate buffer, pH 7.4, 0.3 M sodium chloride [PBS]). The blot was then incubated with 0.48 mg/mL Syt II glutathione S transferase (GST) recombinant protein (BBTech, Inc., North Dartmouth, MA). Following the incubation, the blots were washed and incubated with BCIP and NBT for chromogenic development. BSA (50 mg/mL) was used as negative control.

**ELISA:** The binding of HCD-BoNT/B to gangliosides was evaluated using ELISA-based binding analysis as described by Zhou and Singh (2004). The ganglioside GT1b was purchased from Sigma (St. Louis, MO). Eighty μL of GT1b (3 mg/mL) was diluted in 5 mL of 20 mM sodium phosphate buffer, pH 8.0. Fifty μL of this solution was used to coat each well with agitation on a rocking platform at 4°C for 12 h. After coating, 100 μL of 1% BSA in PBS (blocking buffer) was added to each well and incubated for 2 h at 24°C. The plates were then washed 4 times with PBST (phosphate buffered saline, 0.1% Tween 20, pH 7.4). The test compound mixtures (150 μL) were then incubated in the wells. These mixtures contained HCD-BoNT/B recombinant protein (0.2 to 0.3 mg/mL) and different concentrations of test compound (25, 50, 100, 200, and 300 ng/μL respectively). The mixtures were incubated for 1.5 h at 24°C. The controls consisted of buffer only, and HCD-BoNT/B recombinant protein without test compound. Following the incubation, the plates were washed 4 times with PBST. Bound HCD-BoNT/B was detected with a mouse anti-His-Tag antibody (Ab). The antibody (2 μL) was diluted in 10 mL blocking buffer (1:5000) from which 150 μL was added to each well and incubated for 12 h with agitation at 4°C. In addition some wells were tested with 10 μL of anti-Type B neurotoxin heavy chain BD polyclonal Ab in similar volume of blocking buffer. After the incubation the plates were washed 4 times with PBST. Primary Ab detection was performed by adding 150 μL of a goat anti-mouse IgG-Alkaline phosphatase (AP) conjugate (for His-Tag)

diluted 1:5000 in blocking buffer or anti-rabbit IgG-AP conjugate (for heavy chain BD) diluted 1:1000 in blocking buffer. The plates were incubated 1 h at 24°C. The plates were then washed 5 times with PBST. AP enzyme detection was completed by adding 150 μL of BCIP (5-bromo-4-chloro-3'-indolyl phosphate)/NBT (nitro-blue tetrazolium) each diluted 1:250 with Tris buffered saline (TBS). The plates were incubated for 1 h at room temperature with agitation. The OD (optical density) at 490 nm was recorded using a SpectraMax Plus plate reader with SoftMax ProVer 5.4 software (Molecular Devices, CA).

**Cell line:** Human neuroblastoma cell line SH-SY5Y was purchased from ATCC (American Type Culture Collection, Manassas, VA, #CRL-2266).

**Cell-based toxicity assay:** SHSY-5Y cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum as described (Shi et al., 2008). The cells were placed in a 96-well plate, and incubated overnight in a humidified, 37°C, 5% $CO_2$ atmosphere. The cells were uninduced. The cells were treated with increasing concentrations of a compound for 24 and 48 h at 0.1 to 100 μM concentrations. Both dialyzed and non-dialyzed preparations were tested. Dialysis was done utilizing a Harvard Scientific AmiKa dialysis apparatus with 500 kDa MWCO membrane. At the designated end points (24 and 48 h) the cells were washed once with PBS, and then 0.1 mL of media was added. The cell viability test was done by adding 20 μL of AqueousOne Cell Titer reagent (Promega Corp., Madison, WI) per the manufacturer's specifications. The plates were incubated at 37°C, and spectrophotometer readings were taken at 0, 1, 2, 3, and 4 h at 490 nm. The results were normalized to the untreated controls (100%). The data was analyzed using Graphpad Prism version 5 for determination of the half maximal inhibitory concentration ($IC_{50}$) by the sigmoidal dose response function or linear regression when the response was non-sigmoidal.

**RESULTS**

The crystal structure of BoNT/B complexed with an analog of trisaccharide (PDB_ID: 1F31 http://www.rcsb.org) was used for the *in-silico* screening. We removed the trisaccharide and all the water from the structure and identified four potential binding pockets (Pocket-1, Pocket-2, and Pocket-4) as shown in Figure 1. We targeted these pockets for pharmacophore analysis to discover/design inhibitors against the binding domain of the BoNT/B serotype. For this model, Pocket-1 and combined Pockets-1 and -2 were analyzed (see Fig. 1). Pocket-3 was not considered because of its distance from the GT1b binding site. Pocket-4 has been identified as the Syt protein binding site (Rummel et al., 2004). The five-point pharmacophore model developed for the combined Pockets-1 and -2 is shown in Figure 2A. The resultant five-point pharmacophore model consists of one hydrogen bond acceptor (yellow), three hydrogen bond donors or acceptors (magenta), and one hydrophobic center (green). Figure 2B shows the identified pharmacophore points ideally positioned to characterize shape and chemical nature of the binding pocket with the GT1b trisaccharide docked in the reported x-ray crystal structure complex with BoNT/B. Several amino acids, E1188, E1190, H1241, W1262 and Y1263, were found to be very critical for the binding of the trisaccharide (not shown). Our approach was to use *in-silico* screening (Brooijmans and Kuntz, 2003) to screen compounds against Pocket-1 and against the combined pocket formed by Pockets-1 and -2 together (in green as seen in Fig. 1).

**Figure 1. 3D-surface structure of BoNT/B showing GT1b binding Pockets-1, -2, and -4 in the H$_{CC}$ domain.**
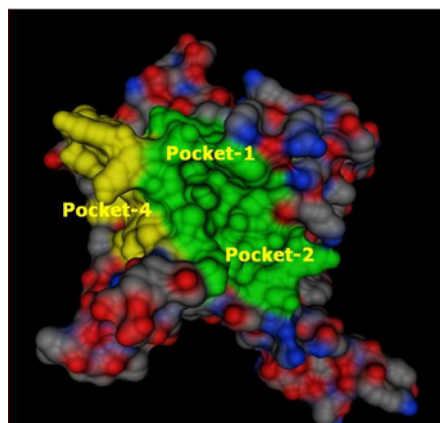


The atoms were color coded (carbon: gray, oxygen: red and nitrogen: blue) on the molecular surface of the C-terminal sub-domain. Pocket-1 (green) is in the ganglioside binding region. Pocket-2 (green) is shown as an extension of Pocket-1. Pocket-4 is shown in yellow as a separate pocket, which was identified by Rummel et al. as Syt protein binding site (structure modified from Rummel et al., 2004, JBC 279:30865-30870). Pocket 3 is not shown.

Six HCBTBD batches were prepared and screened for relative binding activities to Syt II using the immune-dot blot assay (data not shown). Batches 2-5 were found to retain their Syt binding properties, but batches 1 and 6 were below detectable limits. Positive batch preparations with the highest yield of active protein were used in subsequent ELISA assays.

The NCI database of ~120K compounds was filtered, and various conformations for all these compounds, as well as the tautomeric forms of these compounds, were generated using the program MOE (Chemical Computing Group, Montreal, QC, Canada). We generated ~5 million conformations for our *in-silico* screening. Queries were used to search the database for compounds that matched with at least three of the five pharmacophore points. The identified compounds were docked into the site of Pocket-1 and rank ordered using MOE. The protein pocket was kept rigid. Using a consensus scoring function, top-ranking compounds were identified, and the entire complex was energy minimized using MOE. For each screening, consensus scoring based on interaction energy between the compound and the residues in the pocket and surface-based solvation was used to rank order docking of these compounds. Based on the rank ordering of these compounds, the top 100 were identified. After visually checking the location and orientation of the compounds in the binding Pocket-1, 39 potential small molecule compound inhibitors were selected (Appendix 1). Twenty-five compounds selected for Pocket-1/Pocket 2 interactions are shown in Appendix 2. From the two lists, 34 compounds were not available in sufficient quantities for our studies. Thirty compounds were obtained from the NCI. For practical use the compounds need to be soluble in aqueous systems. We initially prepared compounds in 100% DMSO and then diluted into the ELISA buffers. We found 10 compounds insoluble in DMSO, and 7 compounds came out of solution when added to the ELISA buffer. The remaining compounds were tested in the ELISA. All compounds were tested at concentrations ranging from 50 ng/μL to 100 ng/μL. The inhibition testing results for these compounds are shown Table 1. Twelve of 13 compounds appeared to inhibit Syt binding to some degree. Of the Pocket-1 compounds tested (147850, 212147, 280057, 293360, 343982, 403500, and 512796), only 403500 had an inhibition greater than 50% (Table 1). Of the Pocket -2 compounds tested (53924, 112087, 210359, and 211110, 519796, and 522616) only 112087 had an inhibition greater than 50% (Table 1). The highest inhibition of Pocket-1 was by compound 403500 (66.1%) and of Pocket-2 was compound 112087 (65.8%), and the structures of these two compounds are shown in Figure 3.

**Figure 2. Pharmacophore (5-point) model. A.** Model shown in stick form with identified pharmacophore sites (one hydrogen bond acceptor [yellow], three hydrogen bond donors or acceptors [magenta], and one hydrophobic center [green]). **B.** identified pharmacophore points positioned to characterize the shape and chemical nature of the binding pocket with the GT1b.

A



B
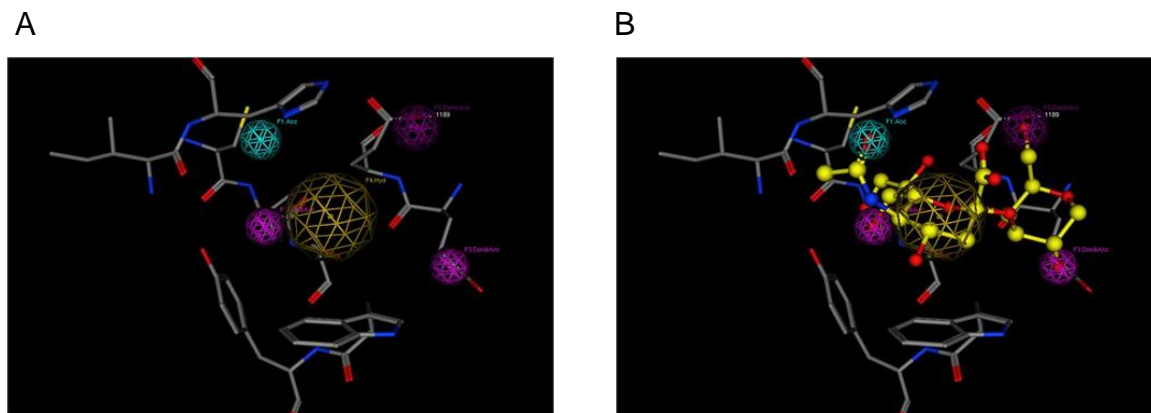


Using 112087 and 403500, we further refined our use of molecular docking and simulations. Comparison of binding sites of both compounds 112087 (Figure 4A) and 403500 (Figure 4B) clearly demonstrates interaction with the binding site via stacking interactions and hydrophobic interactions. However, 403500 (Figure 4B) forms three hydrogen bonds with H1241, Y1263 and N1105 and makes "T-type" stacking with W1262.

Of the 13 selected compounds we tested 11 for toxicity in the neuroblastoma cell line SH-SY5Y (Table 2). Although most of the compounds were found to show some toxicity, three compounds were found to be relatively non-toxic: 211110, 519796, and 522616 with inhibition values of 0.976, 0.841, and 0.667 mg/mL respectively. Compound 403500 was found to have an $IC_{50}$ of 0.0378 mg/mL, and compound 112087 the IC50 was 0.007 mg/mL.

**Table 1** Compound inhibition of HCD-BoNT/B binding to GT1b in ELISA assay. Each compound was tested in triplicate, and the average of three is shown. Compound concentrations were 25 to 300 ng/µL. The Pocket targets are as indicted. ELISA grouping indicates same plate and control values for those indicated compounds. Two potential lead compounds with binding inhibition > 50% are shown in red bold.

| Compound | ELISA Group | mean OD | SD | Buffer only mean OD | HCD-BoNT/B only mean OD | Percent binding with compound | Percent reduction in binding |
|---|---|---|---|---|---|---|---|
| 53924 | 1 | 0.245 | 0.005 | 0.046 | 0.36 | 63.3 | 36.7 |
| **112087** | | **0.140** | **0.012** | | | **34.2** | **65.8** |
| 210359 | | 0.223 | 0.007 | | | 57.8 | 42.2 |
| 211110 | | 0.252 | 0.015 | | | 65.3 | 34.7 |
| 212147 | 2 | 0.069 | 0.011 | 0.043 | 0.123 | 51.8 | 48.2 |
| 293360 | | 0.107 | 0.005 | | | 82.7 | 17.3 |
| 280057 | | 0.117 | 0.008 | | | 91.1 | 8.9 |
| 147850 | 3 | 0.233 | 0.004 | 0.044 | 0.249 | 89 | 11 |
| 343982 | | 0.192 | 0.015 | | | 72.7 | 29.3 |
| **403500** | | **0.118** | **0.019** | **0.049** | **0.303** | **33.9** | **66.1** |
| 512796 | | 0.109 | 0.01 | 0.041 | 0.11 | 94.3 | 5.7 |
| 519796 | 4 | 0.250 | 0.018 | 0.044 | 0.225 | 100 | 0 |
| 522616 | | 0.204 | 0.009 | | | 86.2 | 13.8 |

**Table 2** Compound toxicity for human neuroblastoma SH-SY5Y cell line. Sigmoidal Dose Response analysis except for * analyzed by linear regression.

| Compound | 112087 | 343982 | 280051 | 512796 | 331756 | 519796* | 522616* | 53924 | 211110* | 293360 | 403500 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IC50 (mg/mL) | 0.007 | 0.202 | 0.015 | 0.068 | 0.011 | | | 0.122 | | 2.654 | 0.038 |
| R2 | 0.854 | 0.892 | 0.913 | 0.940 | 0.825 | 0.094 | 0.498 | 0.861 | 0.103 | 0.788 | 0.864 |
| slope | | | | | | -0.006 | -0.024 | | -0.005 | | |
| Y intercept | | | | | | 0.841 | 0.677 | | 0.976 | | |

* Linear regression analysis.

**Figure 3**. **2-D chemical structures of potential lead compounds 112087 (A) and 403500 (B) as represented in National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) repository database.**

A. B.
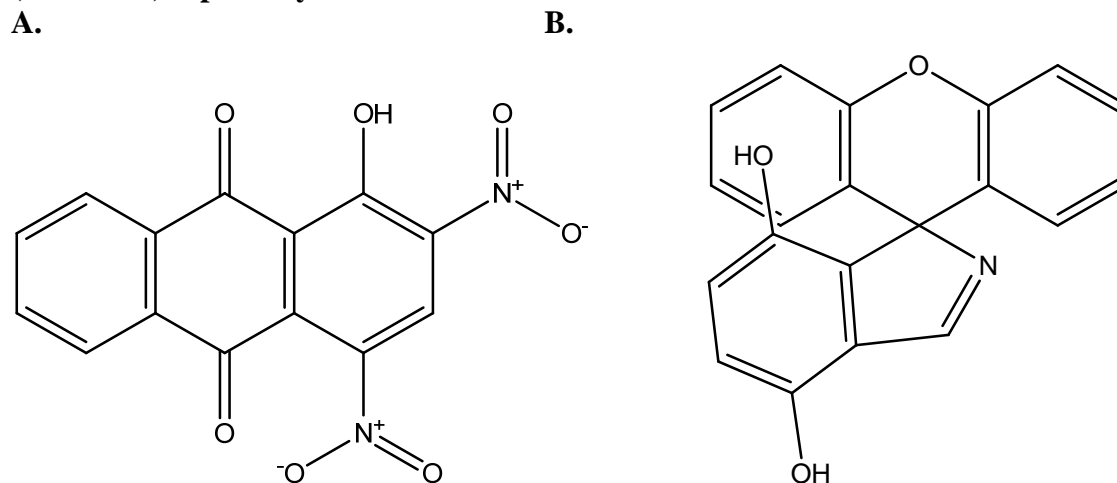


## DISCUSSION

Currently, the only available pretreatment for botulism poisoning exists in the form of a trivalent (A, B, E) botulinum antitoxin that is derived from equine sources utilizing whole antibodies. This antitoxin is only available from the local health department via the CDC in the USA. However, its supply and shelf-life are limited. More recent work by Wang et al. (2010) identified a neutralizing antibody against botulinum neurotoxin B that recognizes the protein receptor binding site for Syt II. While this may prove effective it is also an antibody and has similar limitations as the equine antitoxins. We, therefore, investigated alternative approaches. Here we proposed a strategy of developing small molecules as inhibitors that would block the binding of botulinum neurotoxin to neuronal cells. Identification of such compounds may alleviate the problem of limited shelf-life and availability of the anti-toxin(s) or provide an adjunct treatment to the anti-toxin(s). We developed a five-point 3D stereo-electronic pharmacophore *in-silico* model to the proposed binding pockets of BoNT/B to neuronal cell ganglioside GT1b. *In-silico* approaches have become an integral part of drug discovery and design in academic and industrial research. In recent years *in-silico* screening has been used to discover lead compounds for drug design. It has been used by investigators, for example, Utsintong et al. (2009), to select for anti-cobra toxin drugs. They were successful in finding three lead candidate hits among a library of 1990 compounds from 39 potential inhibitor candidates; using unique pharmacophores they screened compound repositories such as those at http://dtp.nci.nih.gov. For BoNT studies, the application of this approach has led to the development of small molecule non-peptidic inhibitors of the light chain proteases of botulinum neurotoxin serotype A (Tang et al., 2007; Capkova et al., 2009; Burnett et al., 2003; Burnett et al., 2007a; Burnett et al., 2007b; Ruthel et al., 2011; Roxas-Duncan et al., 2009). Roxas-Duncan et al. (2009) discovered small molecule inhibitors against BoNT/A with 92 to 97% inhibition. Burnett et al. (2003) describes small molecule inhibitors against BoNT/A metalloprotease activity. Burnett et al. (2007b) discovered 4-amino-7-chloroquinoline-based inhibitors of the botulinum neurotoxin serotype A metalloprotease using a refined pharmacophore against

BoNT/A.  Burnett et al. (2010) used a three-zone pharmacophore against BoNT/A to screen and discover potential drugs as inhibitors; and others have utilized pharmacophores to help with the discovery and design of inhibitors (Hermone et al., 2008; Nuss et al., 2010).

To generate the pharmacophore model, we examined the GT1b binding pockets (1-4) of the BONT/B HC as shown in Figure 1. Pocket 1 is the ganglioside binding site as identified by binding studies using analogs of gangliosides or saccharides. Pockets 2 and 4 are located close (~13 Å) to the ganglioside binding pocket. Pocket 3 is found at the reverse side at a distance of 28 Å. Since the binding of Syt to BoNT is mediated by the 20 membrane-proximal residues, Pocket 3 is considered to be too far away to mediate the binding.  It should be noted that Pocket 2 is in close proximity to the loosely packed (containing aromatic residues and water molecules) interface region between the N- and C- terminal regions of the binding domain. Chai et al. (2006) reported the structure of the BoNT/B-Syt II complex with a modeled ganglioside that showed a snapshot of how the BoNT/B locks onto the cell surface and is anchored to the presynaptic membrane. Rummel et al. (2004) also reported that BoNT/B has a single ganglioside interaction site within the $H_{CC}$. They reported that Pocket-4 in the $H_{CC}$ binds to Syt and that Pocket-1 in the $H_{CC}$ is the ganglioside binding site (Rummel et al., 2007; Jin et al., 2006). Jin et al. (2006) reported the crystal structure of the complex between BoNT/B and Syt II at a resolution of 2.15-Å and found that Syt-II and the ganglioside bind to two adjacent but non-overlapping binding sites in the $H_{CC}$. In fact Syt I and II have been shown to play a role in the entry of BoNT/B into cells in conjunction with gangliosides (Nishiki et al., 1994; Nishiki et al., 1996; Kitamura et al., 1999; Dong et al., 2003). We utilized the crystal structure of BoNT/B complexed with an analog of trisaccharide (PDB_ID: 1F31) to develop the model shown in Figure 2. We identified a number of amino acids critical for the binding of the trisaccharide: E1188, E1190, H1241, W1262 and Y1263. In our first application we used the model to *in-silico* screen the compound repository of the NCI/DTP of >120k compounds, and over 5 million confirmations were examined. We developed the pharmacophore model to Pocket-1 and -2 as Pocket-4 has been identified as the Syt binding site (Rummel et al., 2004), and Pocket-3 was considered too far for small molecules to reach from Pocket-1 and -2. For Pocket-1 the top 100 compounds were identified, but after visual inspection only 39 compounds were selected (Appendix 1). Similarly, 25 compounds were selected for Pocket-1 and Pocket-2 interactions (Appendix 2). Unfortunately, 34 of these compounds were not available for our studies, and only 30 compounds were obtained from the NCI. Of these 30 compounds, 10 compounds were insoluble in DMSO, and 7 compounds precipitated when added to aqueous solutions, making these impractical for study. The remaining compounds were tested for Syt binding inhibition, and the results are shown in Table 1. In total, 13 compounds were tested, 9 for Pocket-1 and 4 for Pocket-2. Only one compound (519796) targeted to Pocket-1 did not appear to inhibit Syt binding. For Pocket-1 the greatest inhibition was by compound 403500 (66.1%), and for Pocket-2, compound 112087 (65.8%). For the remaining compounds the inhibition varied from 5.7 to 48.2% for Pocket-1 and 34.7 to 42.2 for Pocket-2. These results were used for QSAR refinement of the model, and the refined model is shown in Figure 5.

Only 11 of the compounds were available in sufficient quantity for neuronal toxicity testing. The compounds were tested against the neuroblastoma cell line SH-SY5Y as shown in Table 2. The two best inhibitor compounds identified, 403500 and 112087, had the lowest IC50 values of

0.038 and 0.007 mg/mL respectively. The other compound IC50 values ranged from 0.015 to 2.65 mg/mL. Likely compound optimization would be needed to reduce toxicity.

**Figure 5**. **Model refinement of the binding of compounds 112087 (A) and 403500 (B) in Pocket-1 of the H$_{CC}$ of the BoNT/B.**
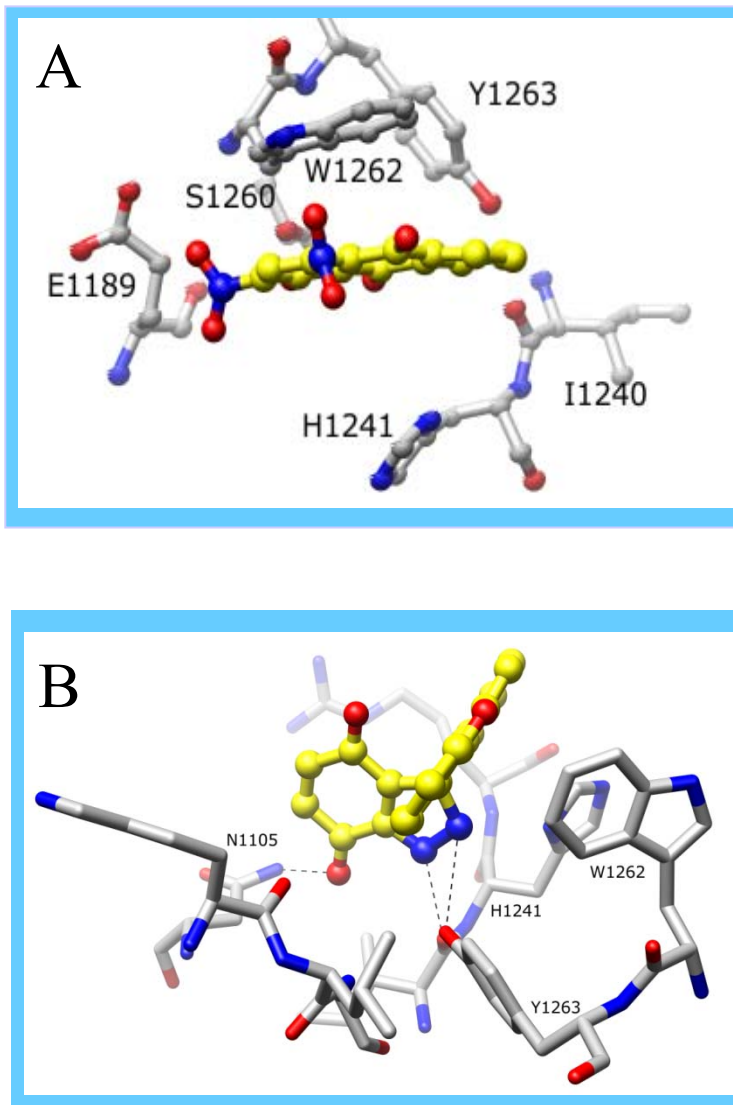


For disease progression, neuronal cell uptake of the toxin is absolutely necessary, and disruption of the process could keep the toxin in circulation longer and available for clearing by the body. Our approach deviates with past approaches to focus on blocking binding of the toxin to the target cell to render BoNT harmless. This strategy could potentially be utilized with all seven neurotoxin serotypes. Therefore, in this investigation we identified potential small molecule inhibitors against the binding of BoNT/B to neuronal cells, using molecular modeling coupled with *in-silico* screening of NCI databases of small commercially available compounds combined with a technique involving the design of a 3D stereo-electronic pharmacophore. A

pharmacophore was designed based on the amino acids forming a binding pocket within BoNT/B HC and utilized to search databases of diverse chemical compounds for molecules that share at least 75% of features located within a given cutoff distance from each other. In this report, we present the results of 3D stereo-electronic pharmacophore based *in-silico* screening of potential inhibitors against the Pocket-1 (ganglioside binding site) of BoNT/B. We developed a five-point pharmacophore (which consisted of one acceptor center, three centers that could be either a donor or an acceptor, and a hydrophobic center) for Pocket-1 on the heavy chain C-terminal of the binding domain of BoNT/B. Docking studies with this pharmacophore by *in-silico* screening of NCI/DTP databases identified 70 small molecule compounds as potential inhibitors against Pocket-1 and the combined Pockets-1 and -2. Two of the compounds have been identified as potential lead candidates, 403500 from Pocket-1 and 112087 from combined Pockets-1 and -2, with inhibition rates of ~66%. While suggestive, further work would need to be done to confirm the binding of these compounds to the BoNT pockets. This may be possible with radiolabeled gangliosides used in competition studies.

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ABBREVIATIONS:

AP, alkaline phosphatase
BCIP, 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
HCBTBD, heavy chain botulinum toxin binding domain
BD, binding domain
BoNT/B, botulinum neurotoxin serotype B
BSA, bovine serum albumin
DMSO, dimethyl sulfoxide
Glu, glutamic acid
HC, heavy chain domain
HCD-BoNT/B, heavy chain neurotoxin type B translocation and binding domains (plasmid)
HCl, hydrochloric acid
$H_{CN}$, heavy chain N-terminal sub-domain region of the BD
$H_{CC}$, heavy chain C- terminal sub-domain region of the BD
LC, light chain domain
NBT, nitro-blue tetrazolium chloride
NCI/DTP (National Cancer Institute/Developmental Therapeutics Program) databases
NIH, National Institutes of Health
QSAR, Quantitative Structure Activity Relationship
Syt, synaptotagmin
TBS, Tris buffered saline
TD, translocation domain